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(54) Title: DETECTION OF POINT MUTATIONS IN *NEU* GENES

(57) Abstract

Oligonucleotide probes reactive with regions of *neu* oncogenes of mammalian origin in which the mutation causing activation of such oncogenes is contained are described, as are methods for their use in detecting the presence of *neu* oncogenes in tumor cells. Antibodies specific for gene products encoded by *neu* oncogenes are also described.

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Detection of Point Mutations in NEU Genes

Description

Background

An increasing body of evidence implicates
5   somatic mutations as causally important in the
induction of human cancers. These somatic mutations
may accumulate in the genomes of previously normal
cells, some of which may then demonstrate the
phenotypes associated with malignant growth. Such
10   oncogenic mutations may include a number of dif-
ferent types of alterations in DNA structure,
including deletions, translocations and single
nucleotide alterations. The latter, also known as
point mutations, may frequently intervene in
15   carcinogenesis, in that a variety of mutagenic
chemicals induce such mutations. In addition, such
mutations may occur spontaneously as a result of
mistakes in DNA replication.

Point mutations have been directly implicated
20   in the causation of 10-15% of human tumors. These
tumors carry oncogenes of the ras gene family, which
differ from their normal cellular counterpart
proto-oncogenes by the presence of a point mutation

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at one of several sites in these genes. These
mutations represent qualitative changes in the tumor
cell genome which distinguish these cells from
normal cells and provide a basis for diagnosis of
5   the genetic origin of a tumor under study. Identi-
fication of the mutations that have created active
oncogenes may provide important diagnostic and
prognostic clues for tumor development. For ex-
ample, a number of mutations has been found to alter
10   the 12th codon of the ras oncogenes, causing
replacement of glycine, which is a normally present,
by any of a number of alternative amino acid
residues. Such amino acid substitutions do not have
equivalent effects; some substitutions (e.g.,
15   valine) create a potent transforming allele while
others (e.g., proline) have only a limited effect on
cellular phenotype. Thus, the identity of a
particular nucleotide substitution may be a strong
determinant of the behavior of the tumor cell (e.g.,
20   its rate of growth, invasiveness, etc.). As a
result, DNA probes of oncogene mutations have
promise as diagnostic reagents in clinical oncology.
        Such probes are useful, however, only if a
region of a gene of interest in which point muta-
25   tions are likely to occur has been identified.
Unless identification of such a region has been
made, it is impractical to use oligonucleotide
probes of limited size (e.g., 10-20 nucleotides
long) to scan an entire gene, which might well be
30   30,000 or more base pairs long; for example, if a
15-nucleotide probe were used to scan the entire

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length of a gene 33,000 base pairs long, 3000-5000
separate probes would be required.

   Thus, although DNA probes of oncogene mutations
have potential as diagnostic tools, they cannot be
5 used effectively unless a discrete region of muta-
tion in the gene, which is causally related to
activation of the gene's oncogenic function, has
been identified. Without such localization of the
region of mutational activation, use of DNA probes
10 specific for point mutations is impractical.

Disclosure of the Invention

   This invention relates to assessment of cellu-
lar DNAs to determine whether they carry lesions or
alterations in a neu gene which result in the
15 activation of the proto-oncogene, and its conversion
to an oncogene. Oligonucleotide probes specific for
nucleotide sequences of the region in which the
activation mutation(s) resides, which can be used to
determine the presence of an oncogene of the neu
20 family are described, as are methods for their use.

   It has been determined that the rat neu proto-
oncogene, which encodes a protein resembling a
growth factor receptor, is converted into an onco-
gene by a single nucleotide alteration or point
25 mutation. This point mutation was initially seen in
a rat neuroblastoma induced by transplacental
exposure to a carcinogen (e.g., ethylnitrosourea)
and found to affect the amino acid sequence of the
transmembrane region of the p185 encoded by the DNA.

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That is, a valine present in the normal protein is
replaced by a glutamic acid residue.

Oligonucleotide probes used to assay for
similar point mutations suspected to be present in
5   seven additional neu oncogenes, each of which arose
in a separate, independently induced tumor, demon-
strated the presence of the same activating muta-
tions in all seven neu oncogenes. The same amino
acid substitution (glutamic acid replacing valine)
10   resulted in these cells. Further assays with
oligonucleotide probes homologous to the neu gene
demonstrates that the mutagen methylnitrosourea
induces formation of nervous system tumors and
activation of neu genes at the same position as
15   shown to occur in the ethylnitrosurea-induced
tumors.

The human homolog of the neu gene (also known
as c-erbB2 or HER2) is thought to achieve an
oncogenic state through the action of a similar
20   mechanism: alteration of a single nucleotide in the
normal cellular DNA sequence (the proto-oncogene),
resulting in activation of the oncogene. The
activating lesion found can be identified in the DNA
of a variety of spontaneously arising human tumors
25   through the use of oligonucleotide probes con-
structed to be specifically reactive with the region
of the human neu gene corresponding to the region in
the rat neu oncogene known to contain the activating
mutations. Identification in human tumor cells of
30   the activating point mutation responsible for
conversion of the proto-oncogene into the neu

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oncogene can serve as the basis for construction of
oligonucleotide hybridization probes useful in
testing human tumor DNAs for the presence or absence
of point mutations responsible for activation of neu
5   oncogenes. These hybridization probes can be used
in detecting the occurrence of the neu
proto-oncogene and of the neu oncogene in cells and
in determining the profile of oncogene activations
in human tumor specimens. Such oligonucleotide
10   probes are described, as are methods for their use
in detecting the presence or absence of neu onco-
genes in tumor cells. Antibodies specific for the
p185 protein encoded by the neu oncogene, which can
be used to detect the occurrence of the neu
15   oncogene, are also described.

Brief Description of the Drawings
     Figure 1 is a schematic representation of
pSV2neu, created by inserting the neu gene cDNA
indicated into the pSV2 expression vector.
20   Figure 2 presents electrophoretic gel patterns
characteristic of cell lines containing pSV2neu
constructions.
     Figure 3 shows nucleotides 1968 to 2073 and the
predicted amino acid sequence for the normal rat neu
25   gene and the transforming rat neu gene.
     Figure 4 shows the nucleotide sequence of
oligonucleotide probes corresponding in sequence to
a) the wild type (normal), b) the mutant neu version
of the neu gene, c) DNA from DHFR G8, and d) a

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modified version of the mutant neu gene in which
there is a T to G transversion.

Figure 5 shows electrophoretic gel patterns
after hybridization of DNA from neu transfectants
5 with oligonucleotide probes.

Figure 6 shows electrophoretic gel patterns
after hybridization of DNA from tumor cell lines and
normal BDIX DNA with oligonucleotide probes.

Figure 7 shows electrophoretic gel patterns
10 after probing of fourteen transfectants containing
ten independent activated neu genes with either the
oligonucleotide corresponding to the normal gene
(proto-oncogene) (top) or the oligonucleotide
corresponding to the transforming gene (oncogene)
15 (bottom).

Detailed Description of the Invention

It is now known that cellular genes other than
the ras genes can be converted into oncogenes by
point mutations. It has recently been shown that
20 the rat neu proto-oncogene, which encodes a protein
resembling a growth factor receptor, can also be
converted into an oncogene by a single nucleotide
alteration. Thus, it has been shown that the neu
proto-oncogene (i.e., the nucleotide sequence
25 present in the normal genome of normal non-tumor
cells) can undergo mutational activation into its
corresponding oncogene (i.e., the nucleotide
sequence whose expression within a cell causes its
conversion from a normal cell into a tumor cell) by
30 a single nucleotide substitution. This point

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mutation, initially demonstrated in a rat
neuroblastoma induced by transplacental exposure to
ethylnitrosourea, has also been shown to occur in
seven other neu oncogenes, each of which arose in a
5   separate, independently induced by ethylnitrosourea
exposure. Detection of the point mutations relied
on the use of oligonucleotide probes specific for
the region initially shown to contain the activating
mutation. In all cases, the same amino acid
10   substitution resulted: a valine normally present in
the transmembrane region of the encoded p185 protein
was replaced by a glutamic acid residue. This
finding suggests that nucleotide substitution at
only a limited number of sites in the neu
15   proto-oncogene will result in its becoming an active
oncogene.
        Transfectants which arose from DNAs from ten
methylnitrosourea-induced nervous system tumors
which contained activated neu genes have also been
20   shown to have the identical alteration in their DNA
and are presumed to have the same sequence change as
that present in the ethylnitrosourea-induced tumors,
based on their differential hybridization to an
oligonucleotide probe corresponding to the
25   transforming gene. Thus, oligonucleotide probes
homologous to the neu gene have been shown to be
able to detect activated neu genes in tumors induced
by exposure to methylnitrosourea. In addition, this
demonstrates that both mutagens cause activation of
30   neu genes at the same position and that activated
neu genes can arise in both BDIX rats

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(ethylnitrosourea-induced tumors) and Buffalo rats
(methylnitrosourea-induced tumors).

Human versions of the rat neu genes have been
isolated and are also referred to as c-erbB2 or
5   HER2. Yamamoto, T. et al., Nature, 319:230-234
(1986); Coussens, L. et al., Science, 230:1132-1139
(1985). The DNA sequences of both the rat and the
human clones predict a 1260 amino acid protein
product of the neu gene.
10   Mutational activation of the human homologs of
the rat neu genes, referred to here as the human neu
oncogenes, is thought to occur through a similar
mechanism: single nucleotide substitution or point
mutation in the human neu gene. It is highly likely
15 that the single nucleotide substitution occurs in
the region of the human neu proto-oncogene that
corresponds with the region in the rat neu oncogene
shown to contain the activating mutations. It is
possible to determine the presence or absence of the
20 activating lesion in the DNA of a variety of
spontaneously arising human tumors through the use
of oligonucleotide probes specifically reactive
either with regions unique to the neu oncogene or
with regions unique to the corresponding neu
25 proto-oncogene. That is, hybridization probes can
be constructed which will react (hybridize) with a
nucleotide sequence occurring in either a neu
oncogene or its corresponding proto-oncogene (but
not in both). Such probes can be used to test human
30 tumor DNAs for the occurrence of a point mutation
responsible for activation of the neu oncogene. For

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example, radiolabelled oligonucleotide probes can be used in the Southern blot hybridization procedure to detect activation of the neu oncogene in human tumors of clinical interest.

5   Because the p185 proteins encoded by neu oncogenes are different from those encoded by their corresponding proto-oncogenes, it is also possible to develop serological reagents, such as polyclonal antibodies or monoclonal antibodies, which are

10   specific for the altered or the normal amino acid sequences in such proteins. These reagents can be used to provide highly sensitive tests for the presence or absence of neu oncogenes by detecting the occurence of the mutated or altered gene

15   products they encode.

The approach described has been used success-fully in identifying the point mutation which causes activation of the neu proto-oncogene in DNA from a chemically induced (i.e., ethylnitrosourea-induced)

20   rat neuroblastoma. It has also been used to verify the occurrence of the same activating point mutations in seven other neu oncogenes (each having arisen in a separate, independently (chemically) induced tumor).

25   In addition, the approach has been used to detect the presence in DNAs from methylnitrosourea-induced nervous system tumors of Buffalo rats. It has been demonstrated that all of the transfected neu genes bore the identical alteration as occurs in

30   the DNA from the ethylnitrosourea-induced tumor. The approach described can be used, with

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modification, in identifying point mutations which
cause activation of human neu oncogenes. In
addition, it is possible to develop serological
reagents, such as polyclonal antibodies or
5   monoclonal antibodies specific for the altered or
the normal amino acid sequences of the proteins
encoded, respectively, by a neu oncogene or its
corresponding proto-oncogene. These reagents can be
used to test cells for the occurrence of the encoded
10 protein and thus for the presence or absence of the
neu oncogene.

The neu gene family

Exposure of perinatal BDIX rats to a single
dose of the alkylating agent ethylnitrosourea leads
15 to a high incidence of neuroectodermal tumors.
Rajewsky, M.F. et al., In:Origins of Human Cancer,
Cold Spring Harbor Laboratory, 709-726 (1977);
Rajewsky, M.F., In:Recent Results in Cancer Research
84:63-76 (1983). Up to 95% of animals mutagenized
20 transplacentally after the fifteenth day of gesta-
tion or injected directly with ethylnitrosourea up
to ten days after birth will develop central and
peripheral nervous system tumors after a dose- and
strain-dependent latency time. These tumors and the
25 cell lines derived from these tumors display the
characteristics of a wide variety of neural and
glial cell types. Schubert, D., Nature, 249:224-227
(1974).

DNA isolated from four independently derived
30 tumor cell lines of this type contains activated
oncogenes which can be detected in an NIH 3T3 focus

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forming assay. The majority of oncogenes detected
in this assay have been shown to be genetically
altered versions of one of the three closely related
ras genes (Varmus, 1984). However, the gene trans-
5 ferred from these neuro/gliobastomas is unrelated to
the ras genes and has been designated neu.

    Neu was first recognized to be a distinct gene
by its association with the 185,000 dalton tumor
antigen, p185, which is displayed on the surface of
10 transfected cells. Neu is related in DNA sequence
to the erbB gene, which encodes the epidermal growth
factor (EGF) receptor, and antisera raised against
the EGF receptor show some cross-reactivity with
p185. However, detailed analysis has shown that neu
15 bears only limited homology to erbB and that the two
genes reside on different chromosomes. Schechter,
A.L. et al., Science, 229:976-978 (1985). Thus, the
neu gene is related to, but distinct from, the gene
which encodes the EGF receptor.

20   cDNA clones of the neu oncogene have been
isolated from cell lines transformed by this gene.
Bargmann, C.I. et al., Nature, 319:226-230 (1986).
Human versions of the same gene have also been
isolated and termed variously c-erbB2 or HER2. The
25 DNA sequences of these rat and human clones predict
a 1260 amino acid protein product of the neu gene
which is colinear with and 50% identical to the
predicted amino acid sequence of the EGF receptor.
By analogy to the EGF receptor, the neu product
30 appears to be a transmembrane protein consisting of
a cysteine-rich extracellular region of 650 amino

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acids, a transmembrane domain, and an intracellular
portion of 580 amino acids consisting in part of a
tyrosine kinase domain.

     Biochemical studies of the p185 protein support

5   these conclusions. p185 is glycosylated and acces-
sible to antisera in intact cells, which is consis-
tent with its being localized at the cell surface.
It also has an associated tyrosine specific protein
kinase activity. p185 does not, however, bind EGF

10 and thus appears to be the receptor for an as yet
unidentified growth factor.

     Isolation of clones of normal and transforming
     alleles of the rat neu gene

     Biologically active genomic clones of normal

15 and transforming alleles of the rat neu gene have
recently been isolated. Hung, M.-C, et al., Pro-
ceedings of the National Academy of Sciences,
U.S.A., 83:261-264 (1986). Structural comparison of
these clones revealed no evidence of gross

20 rearrangements, suggesting that subtle genetic
alterations were responsible for activation of the
neu oncogene. Comparable levels of p185 were shown
to be expressed in nontransformed cell lines
containing the normal allele and in transformed cell

25 lines containing the mutant allele, suggesting that
the alteration responsible for the activation of neu
did not lead to deregulation of expression of the
gene. Such results implicate a transforming lesion
within the encoded protein p185, which should be

30 represented in cDNA versions of the gene.

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Comparison of cDNA clones of the normal neu
gene and transforming cDNA clones

To determine the effect of the alteration
responsible for activation of the neu gene, a
5 comparison was made of the previously isolated
transforming cDNA clone, DNAs from three other
ethylnitrosourea-induced activations of the neu gene
and a cDNA clone of the normal allele of neu.

Isolation of a normal neu cDNA clone

10   It was first necessary to isolate a normal neu
cDNA clone. To do so, a cDNA library was con-
structed using RNA from the cell line DHFR G8.
Hung, M.-C., et al., Proceedings of the National
Academy of Sciences, U.S.A., 83:261-264 (1986). The
15 DHFR cell line was made by transfecting a genomic
cosmid clone containing a complete normal neu gene
from the BDIX strain of rat into NIH 3T3 cells.
These cells express high levels of the neu gene
product, p185, and a high level of the neu RNA
20 transcribed from the transfected gene. cDNA clones
were made by the S1 snapback technique, tailed with
dCTP using terminal transferase, and inserted into
dG-tailed pBR322 at the PstI site. Thirty recombi-
nant plasmids reactive with neu probes were iso-
25 lated.

These plasmid clones were compared by restric-
tion mapping to a full length cDNA clone of an
activated neu oncogene. While these normal cDNA
clones have common sequences with the previously
30 identified transforming cDNA clones, none contained
the entire coding region of neu. A clone containing

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the entirety of the neu coding region was con-
structed, however, by in vitro recombination of two
partial, overlapping clones which share a unique
NaeI site. The 5' end of the resulting clone was
5   sequenced to verify the presence of the initiation
codon for the neu-encoded p185 protein.

       This normal neu clone was inserted into the
pSV2 expression vector to create pSV2neuN, as shown
in Figure 1. Mulligan, R.C. et al., Nature, 277:
10   108-114 (1979). A transforming neu cDNA clone
derived from the B104-1-1 cell line, which is a
secondary transfectant of an activated rat neu gene,
was inserted into pSV2 to create a plasmid desig-
nated as pSV2neuT (Figure 1). pSV2neuT was highly
15   active in a focus-forming assay on NIH 3T3 cells or
Rat 1 fibroblasts. This assay measures the ability
of DNA molecules that have been introduced into
cells by the transfection to convert such cells,
growing in monolayer culture to a transformed state,
20   causing descendants of these cells to form a cluster
or focus of morphologically transformed cells in an
area of the cell monolayer. However, when the
normal neu cDNA, also inserted into the pSV2 vector,
was transfected into NIH 3T3 cells using identical
25   conditions, no foci were observed.

       Comparison of p185 production by transformed
       and nontransformed cells
       Cell lines containing the pSV2neuN or pSV2neuT
plasmids were isolated by cotransfection with
30   pSV2neo (referred to hereafter as the neo-r marker)
and selection of G418. Southern, P.J. and P. Berg,

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Journal of Molecular and Applied Genetics, 1:327-341
(1982). Cell lines expressing the pSV2neuT con-
struct were morphologically transformed and refrac-
tile; those containing pSV2neuN were flat and

5 nontransformed in morphology. These cell lines were
metabolically labeled with ^{32}P orthophosphate and
their lysates incubated with a monoclonal antibody
that specifically precipitates the rat neu gene
product. As shown in Figure 2B, lanes c, d, and i,

10 the levels of labeled p185 were comparable in the
transformed and nontransformed cells. These results
are consistent with earlier work suggesting that neu
has been activated by a mutation in the coding
region of the gene rather than one which deregulates

15 expression. Hung, M.-C. et al., Proceedings of the
National Academy of Sciences, U.S.A., 83:261-264
(1986).

    Identification of the DNA sequences responsible
    for neu gene activation

20   Identification of sequences responsible for the
transforming activity of the pSV2neuT clone was
carried out by using recombinants between this clone
and the pSV2neuN clone carrying the normal allele.
The recombinants were constructed by ligation of

25 appropriate cloned DNA segments. Figure 1 shows the
structure of a series of clones which delineate the
region of neu that carries the activating mutation.
The struture of each recombinant clone shown was
verified by restriction mapping. In each case, at

30 least two independent plasmid isolates were tested
for the ability to morphologically transform NIH 3T3

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cells. All recombinant clones were cotransfected
with the neo-r marker and the morphology of the
resulting G418-resistant colonies was scored.
Morphologically nontransformed colonies were tested
5   to ensure that they were expressing structurally
intact p185 protein from the acquired cDNA clones.

Clones pSV2neuF and pSV2neuB, which contain the
first 719 and 1899 nucleotides, respectively, of the
transforming clone fused to the remaining sequences
10   of the normal clone (Figure 1), were not transform-
ing, although they did direct the synthesis of p185.
Clone pSV2neuH, which contained transforming neu
sequences from the 5' end of the gene up to nucleo-
tide 2387 and normal neu sequences thereafter, gave
15   foci upon transfection and yielded transformed
colonies indistinguishable from those generated by
the parental pSV2neuT clone. pSV2neuC was also
transforming. It contained an XbaI fragment from
nucleotide 2337 to nucleotide 3534 of the normal neu
20   gene, which replaced the corresponding portion of
the transforming cDNA. These results indicate that
the normal cDNA and the transforming cDNA differ in
a sequence between nucleotides 1899 and 2337 and
that the presence of this sequence in the transform-
25   ing clone is necessary for transformation.

In order to prove that this sequences is also
sufficient for transformation, the reciprocal
constructs pSV2neuTN and pSV2neuNT (Figure 1) were
constructed and tested by transfection into NIH 3T3
30   cells and Rat 1 fibroblasts. pSV2neuTN contained
the entire transforming cDNA with the exception of

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nucleotides 1899 to 2387, defined by NdeI and BgII
sites, which are derived from the normal neu clone.
pSV2neuNT contained the entire normal neu cDNA
sequence except for the corresponding 488 nucleo-
5 tides, which were replaced by those from the trans-
forming clone. In parallel experiments, pSV2neuNT
gave comparable numbers of foci to pSV2neuT, the
parental transforming clone; pSV2neuTN, pSV2neuN,
and mock transfected controls yielded no foci.
10 These experiments demonstrate that the essential
genetic differences between the normal and trans-
forming clones reside within this 488 nucleotide
fragment.

The data shown in Figure 2 support these
15 conclusions. G418-resistant cell lines isolated by
cotransfection of the pSV2neu plasmids and the
selectable neo-r gene are shown in Figure 2A. Lines
containing pSV2neuTN or pSV2neuN are morphologically
flat and indistinguishable from lines transfected
20 with the neo-r gene alone. In contrast, cells
containing pSV2neuNT and pSV2neuT are highly refac-
tile and very similar to one another in morphology.
These cell lines were metabolically labeled with
^{32}P-orthophosphate and the resulting lysates incu-
25 bated with anti-p185 monoclonal antibody 16.4.
Drebin, J.A. et al., Nature, 312:545-548 (1984).
Figure 2B shows the levels of p185 expressed in
representative cell lines containing pSV2neuN (lanes
c and d), pSV2neuT (lane i), pSV2neuTN (lanes e and
30 f), and pSV2neuNT (lanes g and h). Lysates from
DHFR G8 and B104-1-1 cells (lanes b and j), the

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cells lines from which the normal and transforming
cDNAs were isolated, were also analyzed. Although
individual cell clones show a wide range of p185
levels, it is clear that there are similar ranges of
5   p185 expression in both the normal and transformed
cells. No p185 is found in lines transfected with
the neo-r marker clone alone (lane a). Thus, the
differences in these cells must be accounted for by
intrinsic differences in the properties of the p185
10   proteins that they express.

Definition of the mutation distinguishing the
normal neu allele and the activated neu gene

The complete DNA sequence of the coding region
of the transforming neu cDNA has been determined.
15   Bargmann, C.I. et al., Nature, 319:226-230 (1986).
To define the precise mutation that distinguishes
the two alleles, the DNA sequence of the region
between nucleotides 1899 and 2387 was determined for
the normal neu cDNA. Only a single difference was
20   found between this sequence and that previously
determined for the transforming clone. At nucleo-
tide position 1012 there is an A in the oncogene
clone, while the normal clone carries a T in this
position. As a result, the predicted amino acid
25   present at residue 664 of the encoded p185 is
affected; a valine found in the normal protein is
replaced by a glutamic acid in the oncogenic ver-
sion. Figure 3 shows the DNA and predicted amino
acid sequence of nucleotides 1968 to 2073 for both
30   the normal neu gene and the transforming neu gene.

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The presumed mutation falls within the putative
transmembrane domain of the neu gene product, p185.
        Genomic clones of normal and transformining
alleles of neu have been previously isolated. These
5 clones were used to independently verify the nucleo-
tide difference seen in the cDNAs. Such corrobora-
tive data served to exclude the possibility that the
observed difference in the cDNAs arose during cDNA
cloning. Sequencing of subclones of the genomic
10 versions of the two alleles confirmed that the same
T to A substitution was present in these genomic
clones. This indicates that the mutation, a T to A
transversion, arose somatically during creation of
the B104 neuroblastoma tumor or cell line.
15   Determination of activation of independent neu
        oncogenes
        Earlier results had shown that DNAs prepared
from four out of six neuro/gliobastoma cell lines
displayed activated neu oncogenes in a NIH 3T3 focus
20 assay. Shih, C. et al.: Nature, 290:261-264 (1981).
These six cell lines had been derived by trans-
placental mutagenesis of BDIX rat embryos with
ethylnitrosourea. These independent activated neu
genes were evaluated to see if they contain the same
25 activating mutation. DNAs from transfectants
containing these neu genes were hybridized with
oligonucleotide probes which would recognize pre-
ferentially one or the other allele of the neu gene.
This technique has been successful in identifying
30 various activated alleles of ras genes. Bos, J.L.

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et al., _Nature_, 315:726-730 (1985); Zarbl, J. _et al._, _Nature_, 315:382-385 (1985).

Oligonucleotides corresponding in sequence either to the wild type or the mutant _neu_ version of
5   the _neu_ gene were synthesized. The sequence of these two 20-mers is given in Figure 4. These 20-mers were then hybridized under stringent conditions (2 degrees below the calculated T_m of a perfect duplex) to dried agarose gels containing
10   DNAs which had been digested with appropriate restriction endonucleases. The 20-mer corresponding to the wild type sequences hybridized approximately ten times as well to pSV2neuN as it did to pSV2neuT. In contrast, the oligonucleotide whose sequence
15   derived from the transforming allele preferentially hybridized to pSV2neuT by the same factor.

DNA was isolated from transfectants carrying the four independently activated _neu_ oncogenes described above and cleaved with HindIII. The
20   resulting fragments were resolved on a 1% agarose gel. The agarose gels were incubated under conditions identical to those used in analysis of the cloned DNAs described above shown in Figure 4. DNA from DHFR G8, which contains about 50 copies per
25   genome of the normal genomic _neu_ gene, was included as a control.

Figure 5A shows hybridization of the transfectant DNAs with the oligonucleotide corresponding to the wild type sequence. A strong signal appears in
30   DHFR G8 DNA, which has introduced copies of the normal _neu_ gene (lane b). Considerably weaker

signals can be seen in lanes containing the neu-
transformed transfectant DNAs (lanes c-f) and in
untransfected NIH 3T3 cells (lane a). Figure 5B
shows an identical gel prepared in parallel probed
5   with the oligonucleotide containing the mutant
sequence. DHFR G8 DNA reacts only weakly with this
probe (lane b) but all neu-transformed transfectant
DNAs (lanes c to f) yield strong signals with this
probe. The transfectant DNA shown in lane d has a
10 smaller neu-homologous HindIII fragment than the
other transfectants due to truncation of the trans-
fected gene at the 3' end.

A low level of reactivity between the wild type
probe and the transfectants is probably due to
15 cross-reactivity of the probe, since it is compar-
able to the signal seen upon analysis of cloned
pSV2neuT DNA with the same probe (compare Figure 4
lanes b and d with lanes c-f in Figure 5A and 5B).

In order to control for differences in signal
20 intensity due to DNA loading and transfectant copy
number, the gel in 5B was stripped of probe and
rehybridized with an oligonucleotide probe from a
different part of the neu gene. The transfectant
cell lines may contain different copy numbers of the
25 rat neu gene due to variable amplification during
the process of transfection. The results of this
hybridization are shown in Figure 5C. Comparison of
corresponding lanes in Figures 5A, B, and C shows
that DNAs of all neu oncogene transfectants exhibit
30 stronger hybridization with the mutant probe than
with the wild type probe, and that the extent of

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their hybridization with the mutant probe correlates
well with the copy number of the neu gene present in
the various DNAs.

Although these data strongly suggest that all
5   of the activated neu genes studied have alterations
at the same nucleotide position, it was not clear
whether the mutations involved the same T to A
transversion in all cases. A T to G transversion
might give the same pattern of hybridization, since
10   it would cause an A/G mismatch with the wild type
oligonucleotide and a relatively stable G/T mismatch
with the mutant oligonucleotide. To address this
possibility, a third 20-mer was synthesized with the
sequence shown in Figure 4. This oligonucleotide
15   was incubated under hybridizing conditions with the
gel analyzed in Figure 5A after the initial probe
had been removed. This oligonucleotide did not
hybridize preferentially to the transfectant DNAs
under stringent conditions (Figure 5D). Thus, the
20   alteration in each of these activated neu genes is
probably the same T to A transversion.

That the point mutation was generated during
tumorigenesis and thus did not reflect preexisting
polymorphism in the rat genome or activation during
25   the transfection process was also confirmed. DNA
was isolated from BDIX rat liver and from the four
rat tumor cell lines which were derived from BDIX
rats and yielded activated neu genes in a trans-
fection assay. Duplicate gels were probed either
30   with the wild type or the mutant oligonucleotide
(Figure 6). BDIX liver DNA reacts well with the

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wild type but not with the mutant oligonucleotide
(Figure 6, lanes a and f). In contrast, the four
tumor cell lines react only with the mutant oligo-
nucleotide (Figure 6, lanes b-e and i-l). This
5 demonstrates that the T to A transversion at nucleo-
tide 2012 arose in the generation of the tumor or
tumor cell lines. It also seems that the tumor cell
lines are hemizygous or homozygous for the activated
allele of the neu gene. Loss of the normal allele
10 may have occurred during tumorigenesis or in passage
of the tumor cell lines. A similar loss or under-
expression of the normal ras alleles has been
observed in several tumors and tumor cell lines that
contain oncogenic versions of these ras genes.
15 Capon, et al. Nature, 304:507-513 (1983): Guerrero,
I et al., Proceedings of the National Academy of
Sciences, U.S.A., 82:7810-7814 (1985).

Multiple independent activations of neu

Chemical carcinogenesis, though provoked by
20 apparently randomly acting agents, often results in
specific genetic changes in the resulting tumor
cells. Neu appears to be activated by the same
nucleotide change in each of the four of the trans-
forming neu alleles analyzed. Each of these alleles
25 was isolated from a neuro- or glioblastoma that
arose from transplacental exposure to the alkylating
agent ethylnitrosourea. Schubert, D. et al.,
Nature, 249:224-227 (1974). Neu is also activated
at the same residue in four independent nervous
30 system tumors induced by the related alkylating
agent methylnitrosourea.

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Similarly, extensive study of
methylnitrosourea-induced mammary carcinogenesis in
Buf/N rats has shown that among nearly 100
independent resulting tumors, all contain H-ras
5   oncogenes activated by the identical G to A
transition at residue 35. Sukumar, S. et al.,
Nature, 306:658-661 (1983); Zarbl, H. et al.,
Nature, 315:382-385 (1985). Other examples of
specificity of activation include
10   dimethylbenzanthracene-induced papillomas with
activated H-ras genes in genetically susceptible
Sencar mice and thymic lymphomas induced by gamma
irradiation or methylnitrosourea, which yield
transfectable K-ras and N-ras oncogenes,
15   respectively. Balmain, A. and I.A. Pragnell,
Nature, 303:72-74 (1983); Guerrero, I. et al.,
Proceedings of the National Academy of Sciences,
U.S.A., 81:202-205 (1984). The K-ras oncogenes
detected in the former case appear to have the same
20   activating mutation in at least three out of four
tumors examined. It is interesting to note that the
administration of the same mutagen, methyl-
nitrosourea, under different conditions leads to the
specific activation of H-ras in mammary tumors,
25   N-ras in thymic lymphomas, and neu in neurectodermal
tumors. Guerrero, I. et al., Science, 225:1159-1162
(1984).

The carcinogen which induces each of these
tumors must initially inflict widespread damage to
30   the cellular DNA. The final mutation detected is,
however, highly specific. Clearly, strong

biological forces must act during multistep
carcinogenesis to select the outgrowth of cells
bearing the genetic lesions observed in the ensuing
tumors. It appears that only a small proportion of
5   cellular genes can be converted into biologically
active oncogenes. Within one of these genes, only a
few of many possible mutations will yield an
actively transforming oncogene. The relatively rare
mutations which do generate oncogenes are enriched
10 first because they must confer a selective advantage
to the tumor, and later because they can be detected
in the focus-forming assay.

The repeated appearance of a specific lesion in
one tumor type suggests the presence of additional
15 forces selecting among the possible activating
mutations. The nature of the activating mutation
must be strongly influenced by the chemical
reactivities of the carcinogen. For example,
different mutagens used to induce thymomas lead to
20 different activated genes in the resulting tumors.
Similarly, mammary carcinomas induced by
dimethylbenzanthracene contain a different specific
alteration from those induced by methylnitrosourea.

The reactivity of ethylnitrosourea, the agent
25 which induced the described neuroblastomas, allows
it to form a number of different adducts in DNA.
Singer, B. and J.T. Kusmierek, Annual Review of
Biochemistry, 52:655-693 (1982). Among these, an
adduct causing G to A transition mutations is well
30 described. Rajewsky, M.F., Recent Results in Cancer
Research, 84:63-76 (1983). Such mutations have been

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found repeatedly as the activating lesions in the
H-ras oncogenes of mammary carcinomas induced by the
related carcinogen methylnitrosourea. Zarbl, H. et
al., Nature, 315:382-385 (1985). The presently
5   described mutations are, however, T to A transver-
sions, the creation of which must be explained by
alternative mechanisms involving another one of the
many adducts formed after administration of these
alkylating agents. This type of mutation is not
10 without precedent: two other ethylnitrosourea-
induced mutations isolated after germ-line muta-
genesis of mice has also been shown to be T to A
transversions. Popp, R.A. et al., Genetics, 105:
157-167 (1983); Lewis, S.E. et al., Proceedings of
15 the National Academy of Sciences, U.S.A., 82:5829-
5831 (1985). An N-ras oncogene isolated from a
methylnitrosourea-induced lymphoma has been found to
be activated by a C to A transversion suggesting
that several different adducts induced by alkylating
20 agents can lead to mutations. Guerrero, I. et al.,
Proceedings of the National Academy of Sciences,
U.S.A., 82:7810-7814 (1985).

Examination of tumor DNAs from Buffalo rats with
oligonucleotide probes homologous to the neu gene.
25   Sukumar and Barbacid (National Cancer
Institute, Bethesda) have isolated ten
methylnitrosourea-induced nervous system tumors of
Buffalo rats which contain activated neu genes.
Transfectants which arose from these tumor DNAs were

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assessed to compare the mutation in these neu genes
to that found in the tumors previously described.

This was carried out in the following manner:
Fourteen transfectants containing ten independent

5   activated neu genes are shown in Figure 7 (tracks
1-14). Duplicate gels were probed with the
oligonucleotide probes described above. That is,
they were probed either with the normal
oligonucleotide (Figure 7, top) or the

10 oligonucleotide corresponding to the transforming
gene (Figure 7, bottom). NIH DNA (track N), DHFR G8
DNA (track G) (high copy of the normal neu gene0,
B104-1-1 DNA (track B) (high copy of the
transforming neu gene) and two Buffalo rat tumors

15 which do not contain active neu genes (tracks c1 and
c2) were included as controls.

All of the transfected neu genes bore the
identical alteration as that in B104-1-1 DNA, the
cell line from which the transforming neu cDNA clone

20 described above was derived. Based on their
differential hybridization to the oligonucleotide
corresponding to the transforming gene, the
transfected neu genes are presumed to have the same
sequence change as the B104-1-1 neu gene. These

25 results provide ten independent examples of
activated neu genes detected with the
oligonucleotide; demonstrate that a second mutagen,
methylnitrosourea, leads to the activation of neu
genes at this position in a similar way as

30 ethylnitrosourea; and demonstrate that activated neu
genes arise in Buffalo rats as well as BDIX rats.

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Oligonucleotide-directed mutagenesis

　　　　Oligonucleotide-directed mutagenesis was used
to substitute a glutamine residue for the valine
residue normally found at amino acid position 664.
5　When this mutated neu gene was inserted into an
expression vector (Psi 2) and transfected into or
infected into recipient (NIH 3T3) cells, 100% of the
stable cell lines generated were transformed.
Korman, A. and R.C. Mulligan, Proceedings of the
10　National Academy of Sciences, USA (April 1987). (In
contrast, 0.1% of recipient cells transformed with
the normal neu gene (proto-oncogene), which encodes
a protein including a value residue at amino acid
position 664, were transformed.) Thus, like
15　glutamic acid, glutamine at position 664 leads to
generation of a transforming neu gene, and mutations
detected with an oligonucleotide probe that would
recognize glutamine would be.presumed to be
transforming mutations. Substitution of an aspartic
20　acid residue at position 664, followed by
transfection or infection into nontransformed cells,
led to the transformed phenotype in 2-3% of the
recipient cells. This appears to represent a weakly
transforming allele of the neu gene, whose
25　overexpression must be coupled to the mutation for
transformation to occur.

Identifying the activation mutation in human neu genes

　　　　Although the discussion to this point, as well
30　as the experimental work described, has been related

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to the point mutation responsible for activation of
the rat neu oncogene, it is possible to use the same
approach in identifying the corresponding activation
mutation in human neu genes and in detecting the
5   presence of a neu oncogene (or its corresponding
proto-oncogene) in human tumor cell DNAs. For
example, oligonucleotide probes can be constructed
which are specifically reactive with the region of a
human neu gene which corresponds to the region shown
10 to contain the point mutation responsible for the
activation of the rat neu oncogene. An example of a
probe which can be constructed is one based on a
single nucleotide difference between a neu oncogene
and its proto-oncogene, this single nucleotide
15 alteration being responsible for conversion of the
neu proto-oncogene into its activated oncogene form.
These probes, which can be of any length, but will
generally be 15 to 20 nucleotides long, can then be
used to analyze a tumor cell genome, to determine
20 whether it carries lesions (mutations) in the neu
oncogene and to determine their precise location, as
described above for the rat neu oncogenes.

Detecting the presence of a neu oncogene
        An assay for detecting carcinogenesis caused by
25 mutation of a neu proto-oncogene into neu oncogene
comprises employing a labelled oligonucleotide probe
specific for a nucleotide sequence present in (or
transcribed from) the proto-oncogene or the
oncogene, but not the other. An assay for
30 carcinogenesis in human cells can be performed by

isolating DNA from the test cells and contacting the
DNA with a labelled polynucleotide probe specific
for either an oncogenic or proto-oncogenic sequence
in the DNA and thereafter determining whether the
5 probe hybridizes to the DNA. After being radio-
labelled, these probes can be used, for example, in
the Southern blot procedure to assess tumor cell
DNAs for the occurrence of such point mutations.
This type of assay can be used in a clinical context
10 as a diagnostic tool to determine the profile of
oncogenes activated in human tumor DNAs. The assay
would be highly specific because it is capable of
detecting single nucleotide alterations in genes of
the neu family, thus providing very definitive
15 information about the tumor cells being assayed.

Reagents for employing these oligonucleotide
probes can be assembled into a kit. Thus, a kit
might contain, in addition to the probe, one or more
buffers, reagents for labelling the probe, reagents
20 employed in Southern or other blots, etc.

Because of the change in amino acid sequence of
the product protein encoded by a proto-oncogene from
the product encoded by an oncogene, it is possible
to detect either by specific serological reagents.
25 The serological reagents can be specific for the
normal, neu proto-oncogene-specified amino acid
sequence at this site of the protein, or be specific
for the altered oncogene-specified amino acid
sequence at this site of the protein. Other sero-
30 logical reagents could be employed that are reacted
with a region of the protein that is unaltered, and

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consequently reactive with either normal or abnormal forms of the encoded protein.

Using cloning techniques, significant amounts of the protein encoded for by the normal site of the
5 proto-oncogene, or by the altered site of the onco- gene, can be isolated. Such protein segments could be used to produce antibodies by standard antibody production techniques. Thus, for producing poly- clonal antibodies, such proteins would be employed
10 to immunize a host, such as a rabbit or a rat, and antibodies to the protein would be collected from serum obtained from the host.

Alternatively, monoclonal antibodies could be produced employing cells which produce antibodies to
15 the protein produced by the isolated gene segment in typical fusion techniques for forming hybridoma cells. Basically, these techniques involve the fusing of the antibody-producing cell with a cell having immortality, such as a myeloma cell, to
20 provide a fused cell hybrid which has immortality and is capable of producing the desired antibody (in this case, an antibody to the normal or altered segment of protein coded for by the isolated gene segment). The hybrid cells are then cultured under
25 conditions conducive to the production of antibody after which antibody is collected from the cell culture medium. Such techniques for producing monoclonal antibodies have been well described in the literature. See, for example, U.S. Patent Nos.
30 4,172,124 and 4,196,265, issued to Hilary Koprowski

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et al., the teachings of which are hereby incorpo-
rated by reference.

Equivalents

Those skilled in the art will recognize, or be
able to ascertain using no more than routine
expermentation, many equivalents to the specific
embodiments of the invention described specifically
herein. Such equivalents are intended to be
encompassed in the scope of the following claims.

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CLAIMS

1. An oligonucleotide probe specifically reactive
    with the region of mutational activation in a
    <u>neu</u> gene of mammalian origin.

5   2. An oligonucleotide probe of Claim 1 which is
        labelled.

3. An oligonucleotide probe of Claim 2 which is
    radiolabelled.

4. An oligonucleotide probe which is specifically
10. reactive with a nucleotide sequence present in
    a <u>neu</u> oncogene of mammalian origin but not
    present in the proto-oncogene for that <u>neu</u>
    oncogene.

5. An oligonucleotide probe of Claim 4 which is
15 labelled.

6. An oligonucleotide probe of Claim 5 which is
    radiolabelled.

7. An oligonucleotide probe comprising from 15 to
    20 nucleotides, the nucleotide sequence of said
20 probe being specifically reactive with a region
    of a <u>neu</u> oncogene of mammalian origin which
    differs from the same region of the proto-
    oncogene of said oncogene by a single nucleo-
    tide, said region of the <u>neu</u> oncogene

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containing the mutation causing activation of
the neu oncogene.

8.   An assay for carcinogenesis in mammalian cells
     caused by mutation of a neu proto-oncogene into
5   a neu oncogene, comprising:
     a.   contacting DNA isolated from the cells
          with an oligonucleotide probe specific for
          a nucleotide sequence unique to either the
          neu proto-oncogene or the neu oncogene,
10   under conditions appropriate for hybridi-
          zation; and
     b.   determining whether hybridization occurs
          between said DNA and said probe.

9.   A method of detecting carcinogenesis in tumor
15   cells, comprising:
     a.   isolating DNA from the tumor cells;
     b.   contacting said DNA with an oligonucleo-
          tide probe, said probe being comprised of
          nucleotides reactive with the region of a
20   neu gene which includes the activating
          mutation, under hybridizing conditions;
          and
     c.   determining whether hybridization occurs
          between said DNA and said probe.

25 10. A method of detecting the presence of a neu
        oncogene in tumor cells, comprising:
        a.   isolating DNA from the tumor cells;

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     b.   contacting said DNA with an oligonucleo-
            tide probe reactive with a nucleotide
            sequence present in a neu oncogene but not
            present in the proto-oncogene for the neu

5   oncogene, under conditions appropriate for
            hybridization; and

     c.   determining whether hybridization occurs
            between said DNA and said probe.

11.   An antibody specific for the gene product

10   encoded by a neu oncogene.

12.   An antibody of Claim 11 which is monoclonal.

13.   An antibody specific for the protein encoded by
      a nucleotide sequence present in a neu oncogene
      of mammalian origin but not present in the

15   proto-oncogene of said neu oncogene.

14.   An antibody specific for the protein encoded by
      a region of a neu oncogene of mammalian origin
      which differs from the same region of the
      proto-oncogene of said oncogene by a single

20   nucleotide, said region of the neu oncogene
      containing the mutation causing activation of
   the neu oncogene.

FIG.I

FIG.2B

FIG.2A

FIG.3
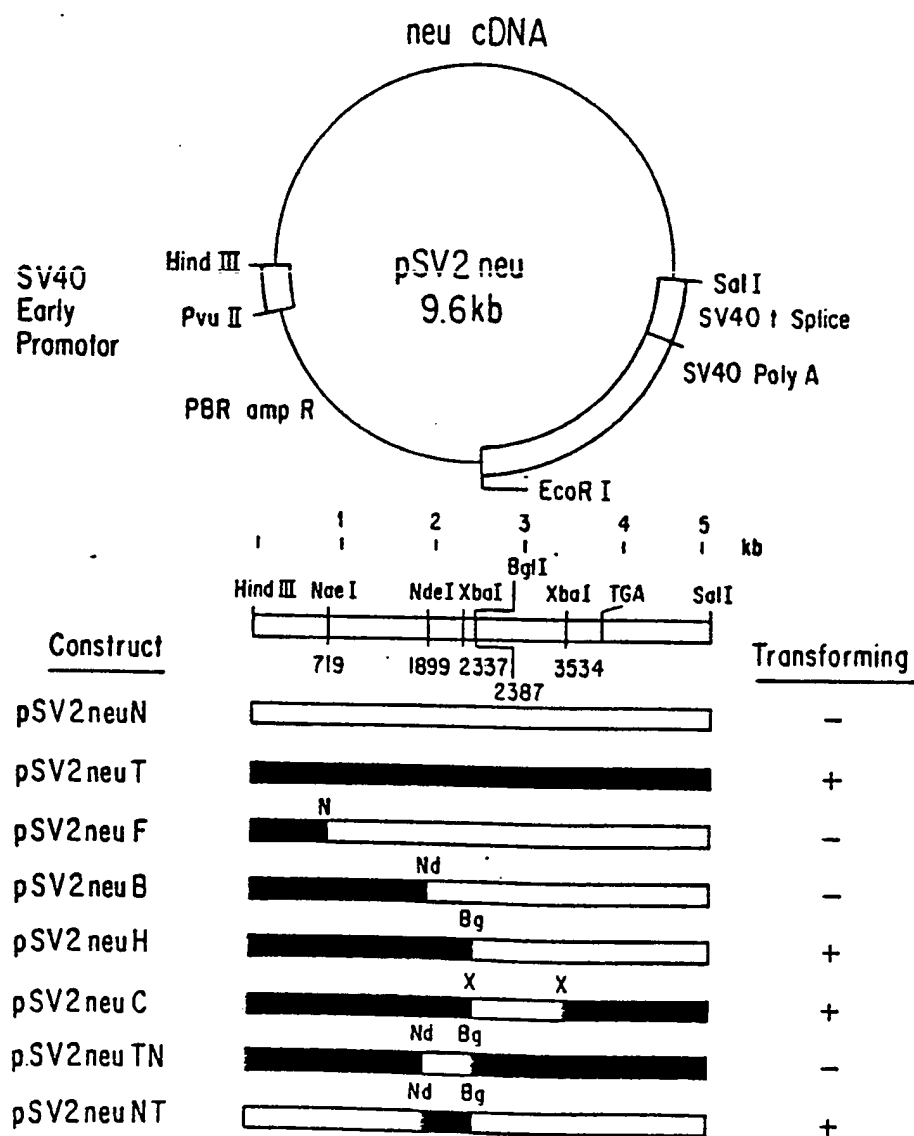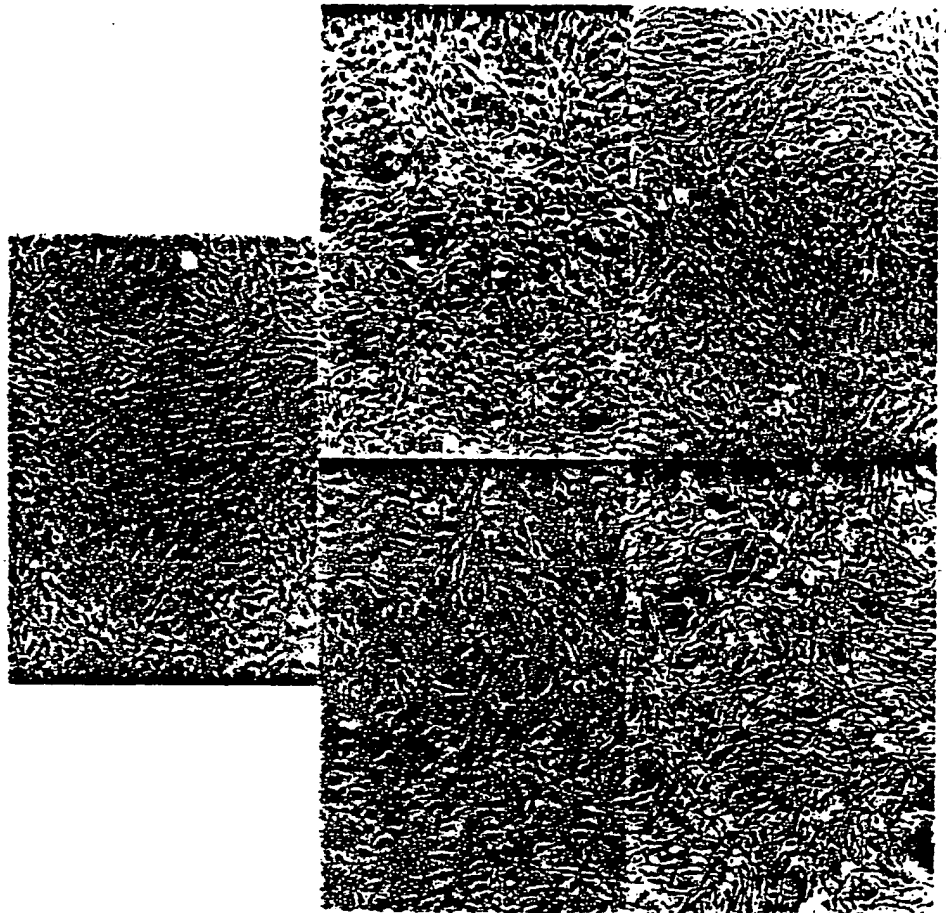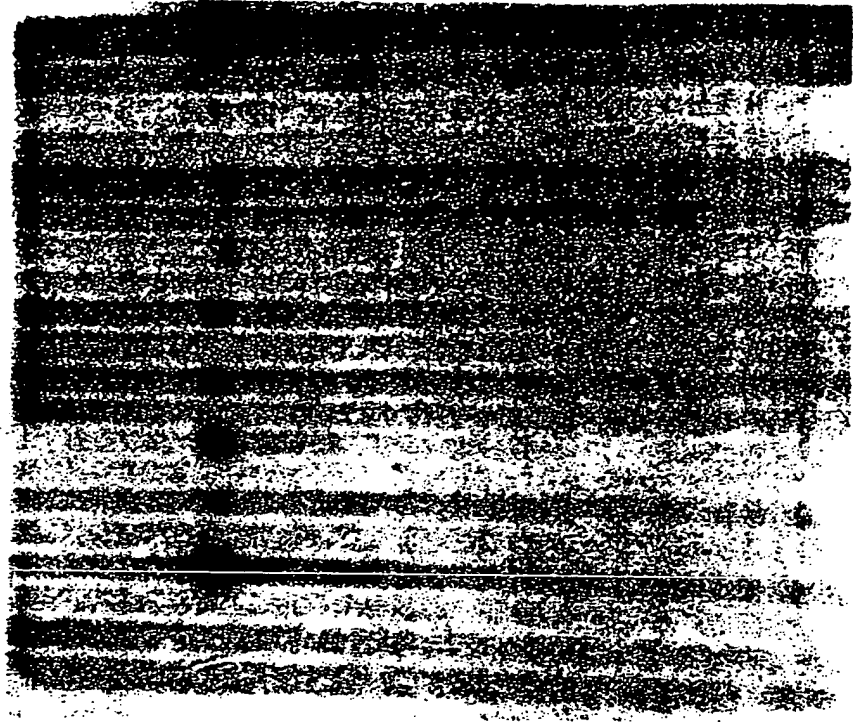
```
normal                                                        val
                                                             GTG
glu gln arg ala ser pro val thr phe ile ile ala thr val  :  gly val     aa 666
GAG CAG AGA GCC AGC CCG GTG ACA TTC ATC ATT GCA ACT GTA  :  GGC GTC
                                                             GAG
transforming                                                 glu


leu leu phe leu ile leu val val val val gly ile leu ile lys arg arg     aa 683
CTG CTG TTC CTG ATC TTA GTG GTG GTC GTT GGA ATC CTA ATC AAA CGA AGG
```

FIG.4

A) ACGCCCACTACAGTTGCAAT   nucleotides 1999-2018, wild-type sequence

B) ACGCCC*TCTACAGTTGCAAT   nucleotides 999-2018, T_{2012} to A

C) CCGTCCTCAGCTGTGACC   nucleotides 996-1013, control probe

D) ACGCCC*CCTACAGTTGCAAT   nucleotides 1999-2018, T_{2012} to G

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FIG.5

FIG.6

a b c d e f g h i j

B
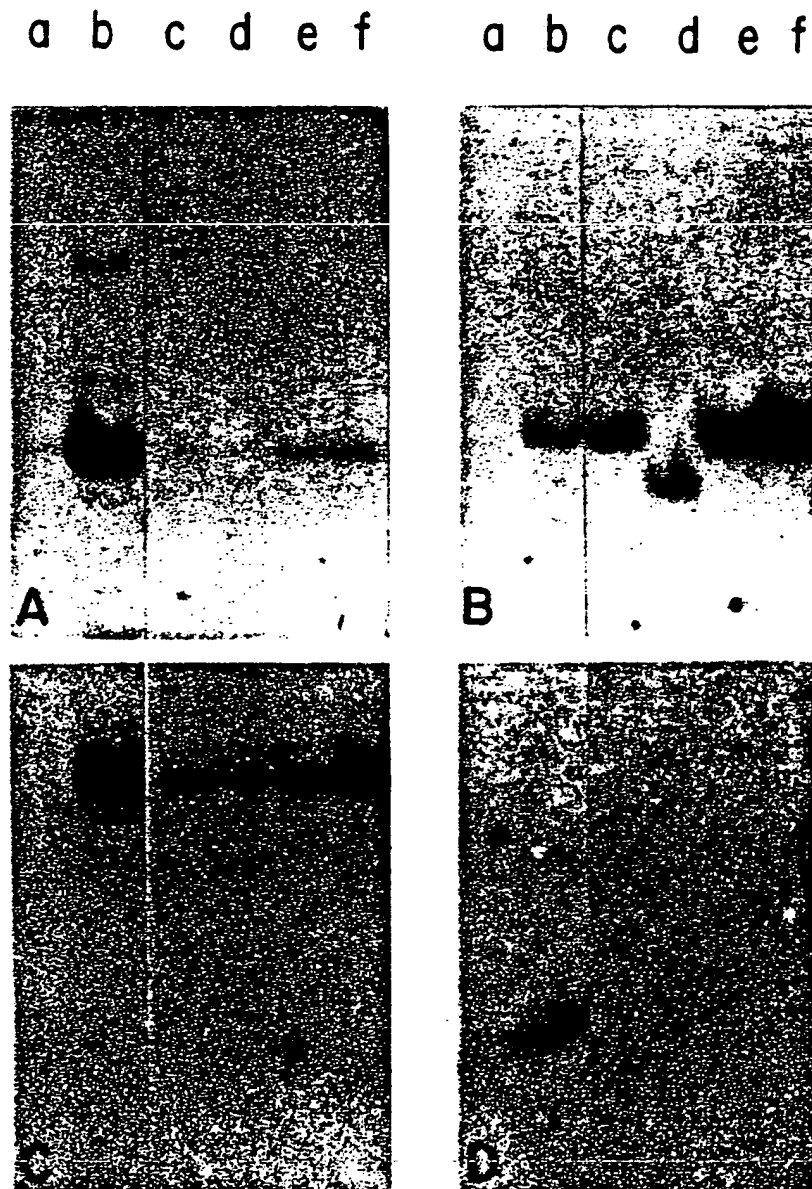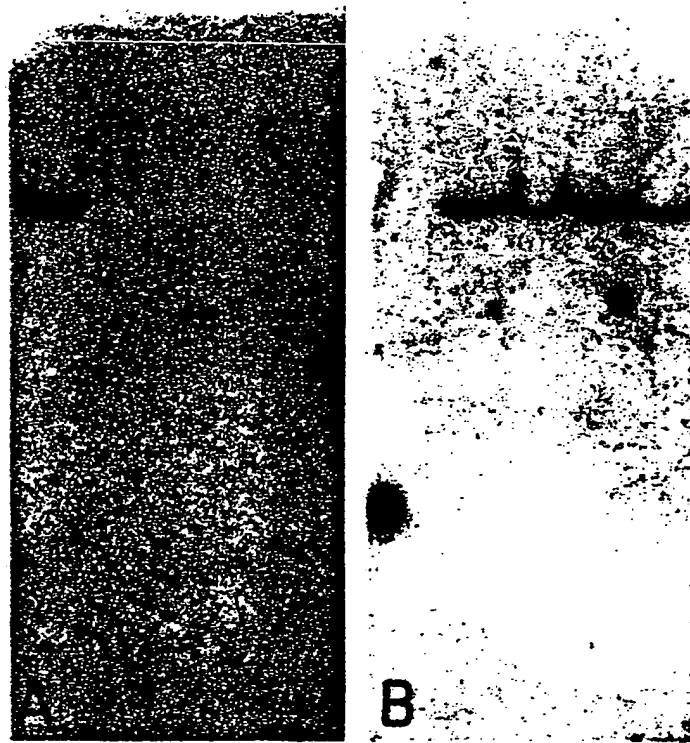
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FIG.7

N G B 1 2 3 4 5 6 7 8 9 10 11 12 13 14 cl c2
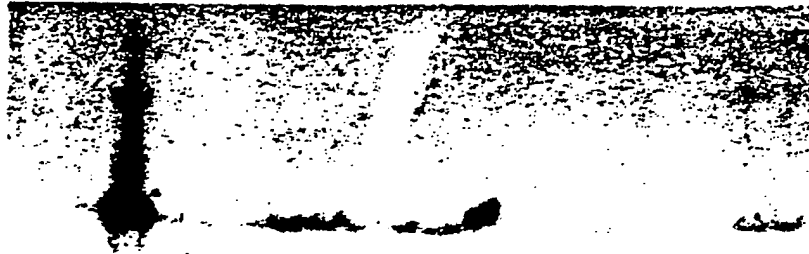
N G B 1 2 3 4 5 6 7 8 9 10 11 12 13 14 cl c2
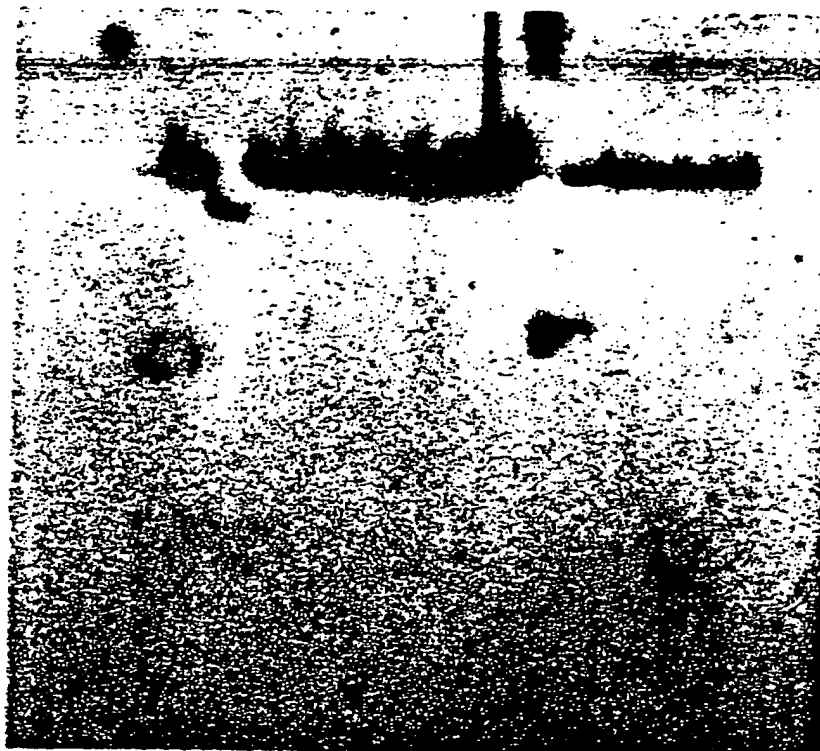
INTERNATIONAL SEARCH REPORT

International Application No. PCT/US 87/01282

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC^4: C 12 Q 1/68; C 12 N 15/00; G 01 N 33/574

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

| Classification System	Classification Symbols
IPC^4	C 12 Q C 12 N G 01 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT *

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
A,P	Chemical Abstracts, volume 105, no. 7, 18 August 1986, (Columbus, Ohio, US), C.I. Bargmann et al.: "Multiple independent activations of the neu oncogene by a point mutation altering the transmembrane domain of p185", see page 162, abstract 55561a, & Cell (Cambridge, Mass.) 1986, 45(5), 649-57	1-10
A	Nature, volume 312, 6 December 1984, A.L. Schechter et al.: "The Neu oncogene: an Erb-B-related gene encoding a 185,000-M_r tumour antigen", pages 513-516 see the whole article	1-6
A	Proceedings of the National Academy of Sciences USA, volume 83, January 1986, M.-C. Hung et al.: "Molecular cloning of the Neu gene: Absence of gross structural alteration in oncogenic alleles", pages 261-264 see the whole article cited in the application	1-10

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the International filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
25th August 1987	11 JAN 1988

International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	M. VAN MOL

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	Nature, volume 319, 16 January 1986, C.I. Bargmann et al.: "The Neu oncogene encodes an epidermal growth factor receptor-related protein", pages 226-229 see the whole article cited in the application	1-6
A	Nature, volume 319, 16 January 1986, T. Yamamoto et al.: "Similarity of protein encoded by the human c-erb-B-2 gene to epidermal growth factor receptor", pages 230-234 see the whole article cited in the application	1-6
A	EP, A, 0108564 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 16 May 1984 see page 1, line 1 - page 8, line 6	1-10
A	Science, volume 225, 14 September 1984, I. Guerrero et al.: "Activation of a c-K-ras oncogene by somatic mutation in mouse lymphomas induced by gamma radiation", pages 1159-1162 see the whole article	1-10

FURTHER INFORMATION CONTINUED FR M THE SECOND SHEET

V.☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1.☐ Claim numbers .... because they relate to subject matter not required to be searched by this Authority, namely:

2.☐ Claim numbers .... because they relate to parts of the international application that do not comply with the prescribed require-ments to such an extent that no meaningful international search can be carried out, specifically:

3.☐ Claim numbers .... because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI.☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ²

This International Searching Authority found multiple inventions in this international application as follows:

    - claims   1-10
    - claims 11-14   (See Form PCT/ISA/206 d.d. 18th September 1987)

1.☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2.☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3.☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers: 1-10.

4.☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (supplemental sheet (2)) (January 1985)

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 09/12/87
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0108564	16-05-84	AU-A- 2065083	10-05-84
		JP-A- 59113898	30-06-84
		AU-B- 559912	26-03-87
		US-A- 4699877	13-10-87

EPO FORM P0479